Reattachment Rate of Human Retinal Pigment Epithelium to Layers of Human Bruch’s Membrane

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Objectives: To determine the reattachment rate of human retinal pigment epithelium (RPE) to different layers of human Bruch’s membrane (BM).

Methods: Explants of BM were prepared from 10 human cadaver eyes by removing native RPE. The RPE basal lamina, inner collagenous layer, elastin layer, and outer collagenous layer were exposed by sequentially removing each apical layer by mechanical or enzymatic means. First-passage human RPE was plated onto the surface and the RPE reattachment rate to each layer of BM was determined.

Results: Retinal pigment epithelial cell reattachment was highest to the inner aspects of BM and decreased as deeper layers of BM were exposed (ie, reattachment rate to basal lamina was higher than to the inner collagenous layer, which was higher than to the elastin layer, which was higher than to the outer collagenous layer). The reattachment rate to the inner collagenous layer, elastin layer, and outer collagenous layer harvested from elderly donors (age >60 years) was less than the reattachment rate to corresponding layers harvested from younger (age <50 years) donors.

Conclusions: Retinal pigment epithelial cell reattachment depends on the anatomical layer of BM present in the host tissue. Age-related changes in BM may interfere with RPE reattachment. Our observations may have implications for understanding the pathogenesis of age-related macular degeneration and its potential treatment with RPE transplantation techniques.

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DURING the last 2 decades, numerous researchers have transplanted retinal pigment epithelium (RPE) into the subretinal space in normal eyes and in eyes with retinal or tapetoretinal degenerations. Typically, these studies have involved delivering suspensions, sheets, or patches of RPE into the subretinal space without removing the native RPE or altering the underlying Bruch’s membrane (BM). The status of the underlying BM has not been considered in many of these investigations, but BM is a complex, multilaminated structure that will affect the reattachment, morphology, differentiation, and subsequent proliferation of transplanted RPE.

We have previously shown that dissociated RPE must reattach to a substrate to avoid apoptosis. These results suggest that the host BM must be able to support the reattachment of donor RPE for the graft to survive. However, BM may be abnormal in patients who are candidates for RPE transplantation, including patients with age-related macular degeneration (ARMD). Thickening of BM and the formation of basal laminar deposits, basal linear deposits, and drusen occurs early in the pathogenesis of ARMD. In addition, surgical removal of subfoveal choroidal neovascularization in ARMD may disrupt the inner aspects of BM, so that the lamellae of BM available for RPE reattachment may not be uniform throughout the transplantation bed. In this article, we address the question of which layers of BM are able to support the reattachment of transplanted RPE, and determine whether the age of the host BM affects RPE reattachment.

RESULTS

Figure 4 shows the ultrastructure of the explants after sequential removal of each layer of BM. Retinal pigment epithelial basal lamina is visible after ammonium hydroxide treatment (Figure 4, A), and the inner collagenous layer is exposed after mechanical removal of the basal lamina (Figure 4, B). Subsequent treatment with...
MATERIALS AND METHODS

PREPARATION OF RPE CULTURES

Retinal pigment epithelial cell cultures were prepared from human cadaver eyes obtained from the Mid-America Tissue Bank (St Louis, Mo) as previously described.22-24 Primary RPE cell cultures became confluent in about 10 days, and cultures were passaged by trypsinization of the cells. First-passage RPE was used in all experiments. Cells were stained using a pancytokeratin antibody to verify that all cells were of epithelial origin as previously described.22,24

HARVESTING OF HUMAN BM EXPLANTS

Explants of human BM were prepared from the periphery of 5 younger (average age, 40.6±11.3 years; range, 23-50 years) and 5 older donors (average age, 72.2±10.1 years; range, 63-89 years) (P=.001). There were no significant differences between the younger and older donor eyes in terms of death-to-enucleation time (5.8±1.9 hours vs 4.1±1.6 hours, respectively; P=.16) and death-to-enucleation preparation time (59.6±30.7 hours vs 57.0±40.9 hours, respectively; P=.91). A full-thickness circumferential incision was made posterior to the ora serrata and the anterior segment and vitreous were carefully removed. The posterior pole of each eye was inspected visually with direct and retroillumination under a dissecting microscope, and globes were discarded if there was any evidence of subretinal blood, extensive drusen, or irregular pigmentation of the macular RPE. The eyes were put in carbon dioxide–free media (Gibco, Grand Island, NY) and a scleral incision was made 3 mm from the limbus and extended circumferentially. Four radial incisions were then made and the sclera was peeled away. A circumferential incision was made into the subretinal space 1 mm posterior to the ora serrata. The choroid-BM-RPE complex was then carefully peeled toward the optic disc and removed after trimming its attachment to the optic nerve. Native RPE was removed by bathing the explant with 0.02 N ammonium hydroxide in a 50-mm polystyrene petri dish (Falcon, Becton Dickinson, Lincoln Park, NJ) for 20 minutes at room temperature, followed by washing 3 times in phosphate-buffered saline (PBS).21 The explant was then floated in carbon dioxide–free media over an unalminated, hydrophobic 125- to 175-µm-thick polytetrafluoroethylene membrane (Millipore, Bedford, Mass) with 0.5-µm pores, with the basal lamina of the RPE facing the membrane. The curiled edges were flattened from the choroidal side with fine forceps, and care was taken to avoid touching BM. We then poured 4% agarose (Sigma Chemical Co, St Louis) on the BM-choroid complex from the choroidal side and the tissue was kept at 4°C for 2 to 3 minutes to solidify the agarose. The polytetrafluoroethylene membrane was peeled off, and 6-mm circular buttons were then trephined from peripheral BM on a Teflon sheet and placed on 4% agarose at 37°C in non-treated polystyrene wells of a 96-well plate (Corning Costar Corp, Cambridge, Mass). The agarose solidified within 2 to 3 minutes at room temperature, thus stabilizing the BM explant. Typically, 6 to 8 explants could be harvested from each eye. A photograph of a typical explant is shown in Figure 1.

PREPARATION OF DIFFERENT LAYERS OF BM

Explants containing different layers of BM on the apical surface were prepared as summarized in the legend for Figure 2. To prepare heparinase- or chondroitinase-treated explants, triplicate buttons from each donor were treated at 37°C with 2.4-U/mL heparinase (Sigma) in PBS at pH 7.5 for 2 hours to remove the heparitin sulfate proteoglycans from the basal lamina of BM, or with 0.1-U/mL chondroitinase ABC (Sigma) in Tris buffer at pH 7.5 for 2 hours to digest chondroitin A, B, C, and dermatan sulfate proteoglycan groups of the basal lamina (Figure 2). Some buttons were treated with heparinase followed by chondroitinase to determine the effects of removing both proteoglycan groups on RPE reattachment. After the enzymatic treatment, the exposed surface was washed 3 times with PBS for 5 minutes. Control explants were washed 3 times with PBS.

The BM explant from the fellow eye was prepared by removing the RPE with 0.02 N ammonium hydroxide as described earlier. The BM explant was then put on a 12- to 18-µm-thick hydrophilic polycarbonate-polysiloxane membrane with 0.4-µm pores (Millipore) with the basal lamina facing toward the membrane. The agarose was allowed to solidify at 4°C, and the hydrophilic membrane was peeled off along with the basal lamina of the RPE, thus exposing the bare inner collagenous layer (Figure 2). Triplicate buttons from each donor were further treated at 37°C with 1-mg/mL collagenase (Sigma) in PBS at pH 7.5 for 1 hour to remove the inner collagenous layer and expose the elastic layer, and with collagenase followed with 20-U/mL elastase (Sigma) in PBS at pH 8.5 for 1 hour to digest the inner collagenous and elastic layers and expose the outer collagenous layer (Figure 2). Some BM explants containing native basal lamina were digested with trypsin to determine the effects of nonspecific proteolysis on RPE reattachment. After the enzymatic treatment, 6-mm-diameter peripheral buttons were trephined and placed on 4% agarose in untreated polystyrene 96-well plates. The wells were gently rinsed with PBS 3 times for 3 minutes, and then stored at 4°C.

collagenase exposes the elastin layer (Figure 4, C), and treatment with elastase exposes the outer collagenous layer (Figure 4, D).

Figure 5 shows the ability of human RPE to attach to different layers of BM 6 hours after plating. The overall RPE reattachment rate to its own basal lamina (53.9%±4.3%) was not altered by treating the basal lamina with heparinase (49.6%±5.11%, P>.05), but was decreased by treating with chondroitinase alone (42.2%±5.6%, P<.01) or chondroitinase with heparinase (34.0%±10.7%, P<.01). The RPE reattachment rate to the inner collagenous layer (37.1%±4.9%, P<.02), elastin layer (21.1%±3.1%, P<.01), and outer collagenous layer (11.0%±8.4%, P<.01) were reduced compared with untreated basal lamina. Extensive digestion of the ammonium hydroxide–treated basal lamina with trypsin markedly decreased the RPE reattachment rate to the surface (9.6%±2.1%, P<.01). Washing the basal lamina 3 times with PBS did not alter cell reattachment significantly (54.4%±12.1%, P>.05). Cell reattachment to tissue culture plastic (41.8%±3.0%) and agarose (0.001%±0%) served as positive and negative controls, respectively.
RPE REATTACHMENT STUDIES

All experiments were performed using RPE cells harvested from a single 52-year-old donor. First-passage RPE cells were used as soon as the cells reached confluence to minimize the effects of culture age on the colorimetric assay described below.26 Confluent cell cultures were synchronized by placing them in serum-free Eagle minimum essential medium for 24 hours prior to harvesting with 0.25% trypsin and 0.25% EDTA in Hanks balanced salt solution for 10 minutes. Two milliliters of 0.1-mg/mL aprotinin (Sigma) in HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (pH 7.5) was added to quench the trypsin reaction, and the cell suspension was centrifuged for 5 minutes at 800 rpm. The cell pellet was washed 3 times and then resuspended in phenol red–free Eagle minimum essential medium without serum. The number of cells was determined by using a Coulter counter (Model Z-1, Coulter Scientific, Hialeah, Fla) and cell viability was assessed using the LIVE/DEAD Viability Kit (Molecular Probes, Portland, Ore).27 This kit contains calcein and ethidium probes and relies on intracellular esterase activity within living cells to cleave the calcein to form an intracellular green fluorescent product. Ethidium passes through the compromised membranes in dead cells and attaches to the DNA, yielding red fluorescence. At least 250 cells were examined under ×100 magnification, and the viability was expressed as the average ratio of live cells to the total number of cells in these 3 different areas.

Aliquots (100 µL) containing 15 000 viable cells were added to the wells containing different layers of BM explants. Positive and negative controls were performed each time the attachment assay was run, with RPE plated on tissue culture plastic serving as the positive control and RPE plated on 4% agarose serving as the negative control. For the controls, cells were allowed to attach to the surface for 6 hours in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Unattached cells were removed from the tissue culture plates by gently washing the wells 3 times with Eagle minimum essential medium.

ASSAY FOR RETINAL PIGMENT EPITHELIAL ADHESION

The number of attached live RPE in each well was determined using a colorimetric assay, which indirectly estimates the number of live cells by measuring intracellular dehydrogenase activity (CellTiter 96 aqueous nonradioactive cell proliferation assay, Promega, Madison, Wis). Dehydrogenase enzymes found in live cells reduce MTS (3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-[4-sulfophenyl]-2H-tetrazolium) into the aqueous-soluble formozan in the presence of an electron coupling agent (phenazine methosulfate [PMS]). The quantity of formozan product can be determined from the absorbance at 490 nm, and is directly proportional to the number of living cells in culture.

The assay was performed in dark conditions owing to the light sensitivity of MTS and PMS. One hundred microliters of Eagle minimum essential medium without phenol red was added to each well; the added solution contained 1.0-g/mL glucose in a bicarbonate-based buffer that maintains the pH at 7.3 to 7.4 in 5% carbon dioxide and 95% air, thus minimizing the effects of changes in glucose and pH on the colorimetric assay.28 Freshly prepared MTS/PMS solution (20 µL, 20:1) was added to each well, resulting in a final concentration of 333 µg/mL of MTS and 25 µmol of PMS. Plates were incubated for 4 hours at 37°C; 100 µL of medium from each well was transferred to the corresponding wells of another 96-well plate and read at 490 nm using an enzyme-linked immunosorbent assay plate reader. The corrected absorbance was obtained by subtracting the average optical density reading from triplicate sets of control sets containing the BM explant on 4% agarose without plated cells. The number of viable cells was estimated from a standardized curve obtained by plating 100 to 20 000 viable, synchronized RPE cells from the same donor in triplicates on BM explants that had been stabilized on 4% agarose. A linear relationship (r=0.93) was observed between the number of viable cells and the absorbance at 490 nm (Figure 3). We also performed the assay for unattached cells (100-20 000) on agarose to demonstrate that cell binding to a surface did not affect the MTS/PMS assay (r=0.97, data not shown).

STATISTICAL ANALYSIS

All experiments were done using BM explants harvested from 5 younger donors (age <50 years) and 5 older donors (age >60 years). The reattachment rate for each well was defined as the ratio of the number of attached viable cells to the number of plated viable cells. Triplicate wells were used to calculate the average reattachment ratio to each layer of BM. Data from all experiments were pooled and expressed as mean±SEM. The attachment rates to different substrates between young and old donors were compared by the Mann-Whitney U test, and the differences between the mean rates of various groups were analyzed in pairs by the Dunn multiple comparison test.29 A confidence level of P<.05 was considered to be statistically significant.

The effect of the age of the donor explant on RPE reattachment rate to each layer of BM was determined by grouping the explants into younger (donor age <50 years) or older (donor age >60 years) explants (Figure 6). The RPE reattachment rate to native basal lamina (younger=54.2%±7.4%; older=53.7%±6.0%; P>.05), basal lamina treated with heparinase (younger=48.2%±8.8%; older=51.0%±6.6%; P>.05), chondroitinase (younger=43.9%±10.7%; older=40.5%±5.12%; P>.05), or heparinase plus chondroitinase (younger=40.5%; older=30.8%±17.7%; P>.05) was not affected by the age of the donor peripheral BM explant. However, the RPE reattachment rate to the inner collagenous layer (younger=47.8%±6.5%; older=26.3%±3.0%; P<.01), elastin layer (younger=29.4%±4.8%; older=12.8%±0.7%; P<.01), or outer collagenous layer (younger=15.9%±4.3%; older=6.0%±0.7%; P<.01) was consistently greater for BM explants harvested from younger eyes compared with those from older eyes. The reattachment rate to trypsin-digested explants was uniformly poor for both age groups (younger=9.1%±3.2%; older=10.5%±1.8%; P>.05).
The reattachment rate of harvested human RPE to different layers of BM is summarized schematically in Figure 7. For all eyes, the RPE reattachment rate is highest if native basal lamina is present on the apical surface of BM. The RPE reattachment rate decreases as deeper layers of BM are exposed, so that the reattachment rate to the basal lamina is greater than the rate to the inner collagenous layer, which is greater than the rate to elastin, which is greater than the rate to the outer collagenous layer. The reattachment rate to the outer collagenous layer is poor, and is comparable to the reattachment rate to trypsin-digested explants. However, we suspect that the outer collagenous layer was partially digested by our enzymatic treatment, since explants were initially exposed to collagenase when the inner collagenous layer was removed.

The age of the donor peripheral BM affects the ability of harvested human RPE to reattach to the inner collagenous layer, elastin layer, and outer collagenous layer. Previous work has shown that the RPE attachment rate to harvested basal lamina was lower when explants were harvested from the macula of older vs younger individuals. Numerous structural alterations develop in each of these layers in the eyes of elderly patients. Abnormal deposits initially develop between the RPE and its basal lamina. Morphological changes subsequently develop in the inner and outer collagenous layer, including deposition of abnormal wide-spaced collagen, vesicular material, amorphous material, and fibronectin. Changes in the ultrastructure of the inner collagenous layer may be responsible for the age-related increase in hydraulic conductivity of BM that develops with advancing age. Calcification and fragmentation of the elastin layer also develop as a function of age.

It is not surprising that ultrastructural changes that develop in BM as a function of age can interfere with RPE reattachment to different layers of human BM. Retinal pigment epithelial cell reattachment to normal human

Figure 1. Photograph of apical surface of the explant surrounded by a ring of agarose (AGR) prior to plating retinal pigment epithelium. Large choroidal vessels are seen deep to the apical surface of Bruch’s membrane (BM).

Figure 2. Schematic describing preparation of different anatomical layers of Bruch’s membrane. Explants containing native retinal pigment epithelium (RPE) on Bruch’s membrane are harvested from human cadaver eyes. Treatment with ammonium hydroxide removes the RPE, but leaves the RPE basal lamina (RPE BL) intact. The basal lamina can be treated with heparinase and/or chondroitinase. The basal lamina can be removed with gentle suction, exposing the fibers of the inner collagenous layer (ICL). Treatment with collagenase removes the inner collagenous layer and exposes the native elastin layer (EL). Treatment with elastase removes the elastin layer and exposes the native outer collagenous layer (OCL). Choriocapillaris basal lamina (CC BL) is present in all preparations.
may decrease expression of RPE binding sites or interfere with binding of fibronectin and other extracellular matrix components to collagen, thus inhibiting RPE reattachment.

Retinal pigment epithelial cell transplantation may become a useful adjuvant in the management of ARMD and tapetoretinal degenerations. Our results suggest that the effects of the disease process on the underlying substrate may affect the initial reattachment of the transplanted cells. The status of the native basal lamina is not known in patients with tapetoretinal degenerations arising from a primary defect in the RPE, but RPE transplantation has been performed after surgical excision of a subfoveal choroidal neovascular membrane in exudative ARMD.34 The ability of transplanted RPE to reattach depends on the ultrastructure of the apical layer of BM re-attachment.
maintaining after submacular surgery, but the cleavage plane that develops during submacular surgery is not known and may not be uniform throughout the surgical bed.19-21 If RPE transplantation is used in nonexudative ARMD, the transplanted RPE may have to reattach to the inner collagenous layer because basal lamina may not be present in areas of RPE atrophy. Our study suggests transplant survival may be affected by the status of the underlining BM.

Treatment with chondroitinase significantly lowered the reattachment rate of RPE cells to their basal lamina, whereas treatment with heparinase did not. The major targets of chondroitinase are chondroitin sulfates and dermatan sulfate, and the major target for heparinase is heparitin sulfate groups in the RPE basal lamina.35 There are several possible explanations for our observations: (1) RPE may contain a receptor for chondroitin sulfates that plays a role in cell reattachment;36 (2) chondroitin sulfates may promote adhesion by acting as a bridge between fibronectin and the collagen framework of the basal lamina;37 (3) chondroitin sulfates may be important in maintaining the 3-dimensional organization of fibronectin, laminin, and collagen in the extracellular matrix;38 (4) chondroitin sulfates may facilitate the binding of integrins to fibronectin; or (5) removing chondroitin sulfates may remove the neutralizing effects of this antibody on tenascin, which is known to inhibit the formation of focal adhesions.39

We recognize that we cannot account for all of the interactions present in the subretinal space in our organ culture studies, including possible effects of the choriorcapillaris, photoreceptors, and cell- or serum-derived cytokines on RPE reattachment and subsequent behavior. However, organ culture studies are the only way to study the attachment of human RPE to normal and abnormal human BM under controlled conditions. In the current study, we used passaged human RPE that were harvested by trypsinization, but the ability of RPE to reattach to each layer of BM may be affected by many variables, including; (1) the technique used to harvest the cells (eg, trypsinization vs treatment with dispase); (2) whether cells are harvested as suspensions, patches, or organized sheets; (3) whether primary or passaged RPE is used; (4) the medium used to grow, transport, and store the cells; and (5) the age of the donor RPE. We are currently performing additional studies to determine the effects of some of these variables on RPE reattachment.

The ultimate success of RPE transplantation will require rapid repopulation of bare areas of BM by transplanted cells, because the choriorcapillaris will undergo atrophy rapidly under areas of BM devoid of RPE.6-17 This will require a thorough characterization of BM at a molecular level in various disease states, and a complete understanding of the interactions that occur between harvested human RPE and normal and abnormal human BM.

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REFERENCES

6. Sheedlo HJ, Li L, Turner E. Functional and structural characteristics of photo-

Figure 7. Schematic summary of the retinal pigment epithelial cell reattachment rate to each layer of Bruch’s membrane.

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